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Isolation of rifampicin resistant *Flavobacterium psychrophilum* strains and their potential as live attenuated vaccine candidates

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ABSTRACT

Previous studies have demonstrated that passage of pathogenic bacteria on increasing concentrations of the antibiotic rifampicin leads to the attenuation of virulence and these resistant strains may serve as live attenuated vaccines. Two rifampicin resistant strains of *Flavobacterium psychrophilum*, 259-93A.16 and 259-93B.17, were generated by passage on TYES plates containing increasing concentrations of rifampicin. Electrophoretic analysis of whole-cell lysates prepared from the parent and resistant strains identified five differentially expressed proteins between the 259-93B.17 strain and parent strain, while there were no differences identified between the 259-93A.16 and parent strain. The LPS banding patterns were identical between all three strains. Bacterial challenges of rainbow trout (*Oncorhynchus mykiss* Walbaum) with the resistant strains demonstrated that the 259-93B.17 strain was highly attenuated and the 259-93A.16 strain was modestly attenuated at the challenge doses tested. Immunization of rainbow trout with the live attenuated 259-93B.17 strain by intraperitoneal injection resulted in significant protection against challenge with the virulent parent *F. psychrophilum* strain at 8 and 15 weeks post-immunization and fish exhibited elevated specific antibody titers. Importantly, immersion delivery of the 259-93B.17 strain stimulated protective immune responses in fish at 10 weeks post-immunization. The results demonstrate that the rifampicin resistant 259-93B.17 strain may serve as an effective live attenuated vaccine for the prevention of *F. psychrophilum* infections.

1. Introduction

Flavobacterium psychrophilum, the etiological agent of bacterial coldwater disease (CWD) and rainbow trout fry syndrome (RTFS), has emerged as one of the most important bacterial pathogens in salmonid aquaculture worldwide [1]. Research efforts are currently aimed at vaccine development for the prevention of CWD. The primary focus has been on killed whole-cell preparations [2–9], subunit vaccines comprised of different protein preparations [10–13], or recombinantly expressed proteins [14,15]. These studies have advanced our knowledge of the *F. psychrophilum* antigens involved in eliciting protective immune responses in fish and provide targets for vaccine development.

An alternative approach to bacterial vaccines comprised of killed cells or purified proteins could include the development of attenuated strains as live vaccines. Numerous experimental live attenuated vaccines have been evaluated with varying levels of protection conferred against different bacterial fish pathogens such as *Yersinia ruckeri* [16], *Edwardsiella tarda* [17], *E. ictaluri* [18–21], *Aeromonas hydrophila* [22,23], *A. salmonicida* [24,25], *Streptococcus iniae* [26], *Vibrio anguillarum* [27], *F. columnare* [28], *Renibacterium salmoninarum* [29], and recently *F. psychrophilum* [30]. Direct and random approaches have been used to induce mutations into these bacterial pathogens to achieve attenuation. Direct approaches include mutation or deletion of genes involved in metabolic pathways and/or pathogenesis [16,17,20,23,25]. Random mutations can be induced using transposon mutagenesis and resultant bacteria are screened for the loss of virulence [22,26,27,31]. Other random approaches include the use of chemicals such as antibiotics [18,27,28,32].

One random approach that has been used to attenuate pathogens for the development of live attenuated vaccines is exposure to rifampicin. Rifampicin is a broad-spectrum antibiotic that inhibits bacterial DNA-dependent RNA polymerase [33] and

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bacteria exhibiting resistance to rifampicin typically have reduced virulence [18,28,34–37]. For example, Schurig et al. [37] reported the development of a resistant strain of *Brucella abortus* (RB51) by repeated passage on media containing increasing concentrations of rifampicin. Immunization of mice with the live RB51 strain conferred protection following challenge with a virulent isolate [37] and this live strain is licensed by the United States Department of Agriculture-Animal and Plant Health Inspection Service Center for Veterinary Biologics (USDA-APHIS-CVB) for the control of *B. abortus* in cattle. This methodology has subsequently been applied to *E. ictaluri* and *F. columnare* and live attenuated vaccines for these fish pathogens have been developed and licensed for use in the United States by the USDA-APHIS-CVB [18,28].

These studies demonstrated the potential for developing live attenuated vaccines for fish pathogens based on the rifampicin resistance strategy. The goal of this study was to apply the rifampicin resistance strategy to *F. psychrophilum*. The specific objectives were to: (1) generate rifampicin resistant strains of *F. psychrophilum* and characterize the protein and carbohydrates of the parent and resistant strains, (2) determine the virulence of the resistant strains, and (3) determine if immunization of rainbow trout (*Oncorhynchus mykiss* Walbaum) with a rifampicin resistant strain confers a protective immune response against *F. psychrophilum* challenge.

2. Materials and methods

2.1. Generation of rifampicin resistant strains

A virulent strain of *F. psychrophilum*, CSF-259-93 [38], was used as the parent strain to generate rifampicin resistant strains. A previously frozen glycerol stock of CSF-259-93 was plated for isolation on tryptone yeast extract salts (TYES; 0.4% tryptone, 0.04% yeast extract, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% $CaCl_2 \cdot 2H_2O$, pH 7.2) agar [39] and incubated at 15 °C for 5 d. A single colony was passed to TYES agar containing 5 μg ml^{-1} rifampicin (Sigma, St. Louis, MO, USA) and incubated at 15 °C for 6 d. Two of the resulting colonies were randomly selected, designated 259-93A and 259-93B, and independently passed to TYES agar containing increasing concentrations of rifampicin. This process was repeated until the 259-93A and 259-93B strains achieved growth at rifampicin concentrations of 200 and 250 μg ml^{-1}, respectively. This required 16 passages for the 259-93A strain (designated as 259-93A.16) and 17 passages for the 259-93B strain (designated as 259-93B.17). Following each passage, a portion of the recovered cells was harvested, resuspended in sterile 20% glycerol, and frozen at −80 °C.

2.2. Bacterial culture

F. psychrophilum strains were cultured for 48–72 h at 15 °C in 200 ml TYES broth for challenges, immunizations, whole-cell lysate production, and carbohydrate extractions. Bacteria were harvested by centrifugation at 4300 × *g* for 15 min and the supernatant was removed. Bacterial pellets were resuspended in sterile phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, pH 7.2) at different concentrations depending on the challenge or immunization trial. The number of colony forming units (cfu) ml^{-1} was determined using a 6 × 6 drop plate method [40], with the exception that TYES agar plates were used and incubated at 15 °C for 96 h.

Growth curves of the CSF-259-93, 259-93A.16 and 259-93B.17 *F. psychrophilum* strains were determined by inoculating duplicate culture tubes containing 20 ml TYES broth with 50 μl of adjusted cultures (optical density of 0.1 at 525 nm) from each strain. The cultures were incubated at 15 °C for 9 d on an orbital shaker (80 rpm) and the growth was monitored daily by measuring the optical densities at 525 nm.

2.3. Analysis of whole-cell lysates and carbohydrate extractions

Whole-cell lysates of each strain were prepared by resuspending approximately 80 mg (wet weight) of cells into 1 ml sterile PBS and sonicating four times for 30 s each at 20% amplitude (Model 500 Sonic Dismembrator, Fisher Scientific, Pittsburgh, PA, USA). Lysates were cooled on ice for 2 min between each 30 s sonication step and were frozen at −80 °C. The protein concentration was determined using a micro BCA protein assay (Pierce, Rockford, IL, USA). Whole-cell lysates (25 μg protein) from each strain were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoreisis (SDS-PAGE) as previously described [11]. Proteins were stained with Bio-Safe Coomassie (Bio-Rad, Hercules, CA, USA) and Precision Plus protein standards (Bio-Rad) were used to estimate the molecular mass of proteins.

The carbohydrates from each strain were extracted according to the method of Hitchcock and Brown [41] as modified by LaFrentz et al. [11]. Undiluted extracts (12 μl) from each strain were analyzed by SDS-PAGE as described by LaFrentz et al. [42]. Gels were stained with PRO-Q Emerald 300 LPS Gel Stain (Molecular Probes, Eugene, OR, USA) to visualize the LPS bands.

2.4. Fish and rearing conditions

Rainbow trout with mean weights of 15 g or 4.6 g were obtained from Clear Springs Foods, Inc. (Buhl, ID, USA) and used in *F. psychrophilum* challenge trials at Clear Springs Foods, Inc. to assess the virulence of the parent and rifampicin resistant strains. Prior to challenge, fish were acclimated for at least one week in 378 L tanks supplied with specific-pathogen-free 15 °C spring water treated with ultraviolet light. Fish were maintained in 19 L tanks during bacterial challenges and fed ad libitum daily with an expanded trout feed (Clear Springs Foods, Inc.).

Eyed rainbow trout eggs were obtained from Clear Springs Foods, Inc. or Hayspur State Fish Hatchery (near Gannett, ID, USA) for use in injection and immersion immunization trials, respectively. Upon arrival, eggs were disinfected with 100 ppm iodophor and were reared according to standard practices using 15 °C dechlorinated municipal water at the University of Idaho (Moscow, ID, USA). Immunized fish were maintained in separate 278 L tanks and fed 2% body weight day^{-1} (Rangen Inc., Buhl, ID, USA). Following bacterial challenges, fish were maintained in 19 L tanks and fed ad libitum daily.

2.5. Assessment of virulence

To assess the virulence of the *F. psychrophilum* strains, a subcutaneous injection challenge model was utilized [3,5,11,43]. Rainbow trout were challenged by subcutaneous injection (25 μl) with the parent CSF-259-93, 259-93A.16 or 259-93B.17 *F. psychrophilum* strains as previously described [11]. In trial 1, duplicate groups of 25 rainbow trout (mean weight, 15 g) were challenged with each strain resuspended in PBS to optical densities of 0.6 or 0.4 at 525 nm. In trial 2, duplicate groups of 25 rainbow trout (mean weight, 4.6 g) were challenged with each strain resuspended in PBS to an optical density of 0.4 at 525 nm or a 1:10 dilution of this suspension. In both trials, a group of mock infected controls ($n = 25$) were injected with 25 μl of sterile PBS.

Mortalities were recorded daily for 28 d and re-isolation of *F. psychrophilum* was attempted as previously described [11]. The

cumulative percent mortality (CPM) was calculated for each strain at the challenge doses tested.

2.6. Immunization trials

2.6.1. Injection delivery

Two groups of 350 rainbow trout (mean weight 2.4 g) were used in the study. Following anaesthetization by immersion into ~90 mg l^{-1} tricaine methanesulfonate (MS-222, Argent Chemicals, Redmond, WA, USA), fish in the treatment group were injected intraperitoneally (ip) using a 30-guage needle with 50 μl containing approximately 8.3×10^6 cfu $fish^{-1}$ of the 259-93B.17 strain and fish in the control group were injected ip with 50 μl of PBS as a mock immunization. At 5 weeks post-immunization, fish in the treatment group were booster immunized by ip injection with 50 μl containing approximately 6.9×10^6 cfu $fish^{-1}$ of the 259-93B.17 strain and fish in the control group were injected ip with 50 μl of PBS.

At 8 and 15 weeks post-immunization, rainbow trout were challenged by subcutaneous injection (25 μl) with the parent CSF-259-93 *F. psychrophilum* strain. At 8 weeks post-immunization, triplicate groups of 25 fish from the treatment and control groups were challenged at two doses, corresponding to 4.5×10^6 and 2.1×10^6 cfu $fish^{-1}$. At 15 weeks post-immunization, triplicate groups of 25 fish from the treatment and control groups were challenged with one dose, corresponding to 1.8×10^6 cfu $fish^{-1}$. In each trial, mock infected controls ($n = 25$ fish per treatment or control group) were injected with 25 μl of sterile PBS. Mortalities were monitored as described above and the CPM was determined for the treatment and control groups at each challenge dose, and relative percent survival (RPS) of the treatment group was determined as previously described [44].

Serum samples were obtained from rainbow trout in the treatment and control groups prior to immunization, at the booster immunization, and prior to both bacterial challenges as previously described [11]. Prior to immunization, serum was collected from 25 randomly selected fish (five pools of 5 fish). At the booster immunization and prior to both bacterial challenges, serum was collected from 30 randomly selected fish (ten pools of 3 fish) from both treatment and control groups.

2.6.2. Immersion delivery

A pilot immersion immunization trial with the 259-93B.17 strain was performed. Three groups of approximately 100 rainbow trout (mean weight, 3.4 g) were used. One group was immunized by immersion into water containing 1.4×10^8 cfu ml^{-1} of the 259-93B.17 strain for 1 h with aeration. The second group was immunized identically with the exception that the adipose fin from each fish was removed just prior to immunization. The control group was mock immunized by immersion into water diluted with sterile TYES media (1:4 dilution) for 1 h. At 4 weeks post-immunization, both treatment groups were booster immunized by immersion into a solution of water containing 9.7×10^7 cfu ml^{-1} of the 259-93B.17 strain for 1 h. The control group was mock immunized as described above.

At 10 weeks post-immunization, duplicate groups of 20 fish from each group were challenged by subcutaneous injection (25 μl) with the parent CSF-259-93 *F. psychrophilum* strain at two doses, corresponding to 2.0×10^6 and 3.3×10^5 cfu $fish^{-1}$. Mock infected controls ($n = 20$ fish per treatment or control groups) were injected with 25 μl of sterile PBS. Mortalities were monitored as described above and the CPM and RPS was determined for each group.

2.7. Enzyme-linked immunosorbent assay (ELISA)

Specific antibody titers against *F. psychrophilum* in serum samples were determined by an ELISA assay. Briefly, pooled serum samples obtained from treatment and control rainbow trout were serially diluted in doubling dilutions from 1:100 to 1:6400 in PBS containing 0.02% sodium azide, applied to 96-well plates coated with *F. psychrophilum* (CSF-259-93) antigen, and specific antibodies were detected as described by LaFrentz et al. [5]. The titer was defined as the reciprocal of the highest dilution exhibiting an optical density of at least two times greater than the negative control. The negative control consisted of a pool of equal volumes of serum sampled from the five, 5-fish pools collected from fish prior to immunization.

2.8. Statistical analyses

The mean CPM data from all challenges were normalized using the arcsine square root transformation [45]. CPM data from strain virulence and immunization experiments were analyzed by one-way analysis of variance (ANOVA) with Tukey's test for pair wise comparisons or Student's *t*-test, as appropriate. Serum antibody titers from immunized and control groups were $\log_{10}$ transformed and then analyzed by a Student's *t*-test. Differences were considered significant when $P < 0.05$. Data were analyzed and graphically represented using GraphPad Prism (version 2.01, GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Growth comparison

Growth curves of the parent CSF-259-93, 259-93A.16, and 259-93B.17 *F. psychrophilum* strains were determined in TYES broth at 15 °C (Fig. 1). The lag and exponential growth phases were similar for all strains from 0 to 3 d post-inoculation. Following 3 d, the rifampicin resistant strains grew at a slower rate than the parent strain and the final cell densities were lower (Fig. 1).

3.2. Protein and carbohydrate characterization

Whole-cell lysates and carbohydrate extractions were prepared from each strain and analyzed by SDS-PAGE (Fig. 2). The 259-93B.17 strain exhibited five differentially expressed proteins, as indicated by changes in banding patterns, when compared to the

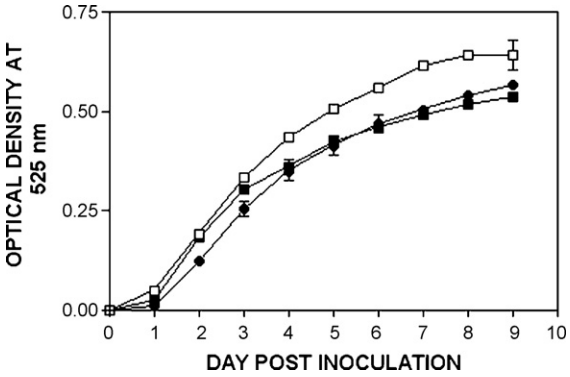

Fig. 1. Growth curves of the parent and rifampicin resistant *F. psychrophilum* strains: (□) parent CSF-259-93 strain; (●) 259-93A.16 strain; (■) 259-93B.17 strain. Duplicate cultures of each strain were grown in TYES broth at 15 °C and growth was monitored by determining the optical density at 525 nm. Error bars indicate the standard error of the mean.

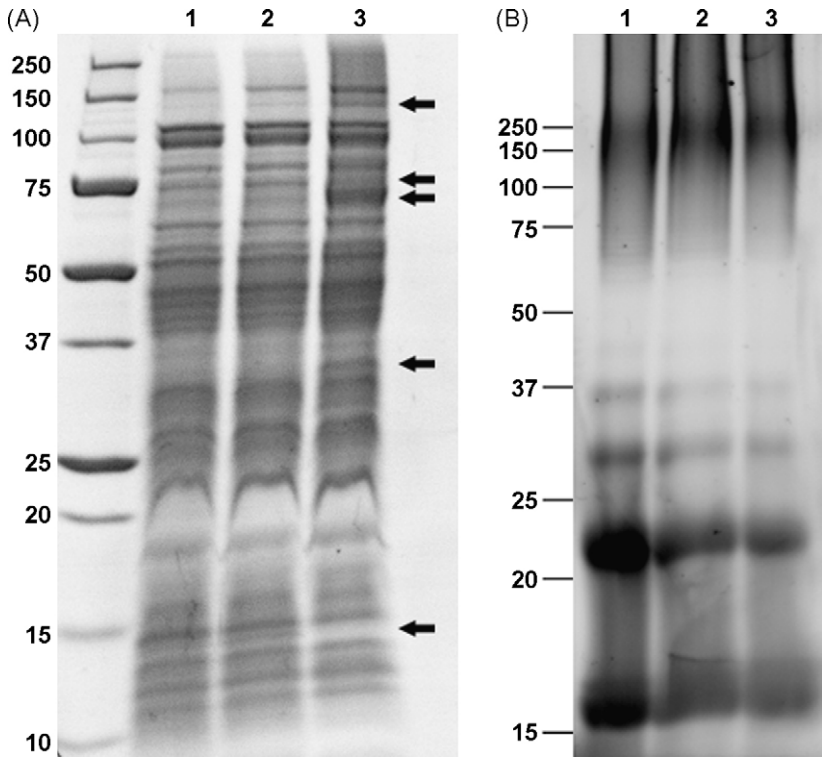

Fig. 2. SDS-PAGE analysis of whole-cell lysates (A) and carbohydrate extractions (B) prepared from the parent *F. psychrophilum* CSF-259-93 strain (Lane 1), 259-93A.16 strain (Lane 2), and 259-93B.17 strain (Lane 3). Whole cell lysate proteins were stained by coomassie and carbohydrate extractions were stained with PRO-Q Emerald 300 LPS Gel Stain. The arrows in (A) indicate protein differences identified in the 259-93B.17 strain. Molecular mass markers (kDa) are indicated on the left of each gel.

parent CSF-259-93 and 259-93A.16 strains (Fig. 2A). The 259-93B.17 strain exhibited increased expression of proteins with approximate molecular masses of 145, 69, and 33 kDa. Additionally, this strain exhibited reduced expression of two proteins with approximate molecular masses of 75 and 14 kDa. There were no differences in protein banding patterns between the parent CSF-259-93 and 259-93A.16 strains. The LPS banding patterns were identical among the three strains although there were minor variations in band intensities (Fig. 2B). All strains possessed five LPS bands with approximate molecular masses of 16, 23, 29, 36, and 43 kDa and also contained minor quantities of glycocalyx carbohydrates, visualized as a ladder of fine repeating bands with molecular masses greater than 60 kDa.

3.3. Assessment of virulence

Challenge of rainbow trout with the parent CSF-259-93, 259-93A.16, and 259-93B.17 *F. psychrophilum* strains demonstrated that the 259-93A.16 was modestly attenuated and the 259-93B.17 strain was highly attenuated (Table 1). At the high dose in trial 1, the CPM of fish challenged with the 259-93B.17 strain was significantly different than the CPM of fish challenged with the parent CSF-259-93 and 259-93A.16 strains ($P < 0.05$), while there were no significant differences between the CPM of fish challenged with the parent CSF-259-93 and 259-93A.16 strains. Similar mortality trends were observed at the lower challenge dose in trial 1 and also in trial 2, which used rainbow trout with a mean weight of 4.6 g (Table 1).

Challenge mortalities exhibited typical signs of *F. psychrophilum* infections including necrotic lesions at the site of injection and eroded/frayed fins. Yellow-pigmented bacteria phenotypically consistent with *F. psychrophilum* were re-isolated from 93% (50/54) and 86% (24/28) of the mortalities examined in trials 1 and 2, respectively. There were no mortalities in the mock infected control groups.

3.4. Injection immunization trial

A protective immune response against *F. psychrophilum* was conferred to rainbow trout following immunization by ip injection with the 259-93B.17 strain (Table 2). Fish given mock immunizations with PBS sustained high CPM (nearly 100%) upon challenge with the parent CSF-259-93 strain, whereas fish immunized with the 259-93B.17 strain exhibited a significantly decreased CPM at both 8 and

Table 1
Cumulative percent mortality (CPM) ± standard error of the mean (SEM) among rainbow trout following experimental challenge with the parent CSF-259-93, 259-93A.16, and 259-93B.17 *F. psychrophilum* strains

F. psychrophilum strain	Trial 1: 15.0 g rainbow trout		Trial 2: 4.6 g rainbow trout	
	CPM ± SEM: OD 0.4	CPM ± SEM: OD 0.6	CPM ± SEM: 1:10, OD 0.4	CPM ± SEM: OD 0.4
CSF-259-93	$66.0^{a} \pm 18.0$	$78.0^{a} \pm 10.0$	$18.9^{a} \pm 2.9$	$54.0^{a} \pm 10.0$
259-93A.16	$28.4^{a,b} \pm 7.6$	$61.4^{a} \pm 9.4$	$2.0^{a,b} \pm 2.0$	$31.2^{a} \pm 0.8$
259-93B.17	$2.0^{b} \pm 2.0^{*}$	$0.0^{b} \pm 0.0$	$0.0^{b} \pm 0.0$	$0.0^{b} \pm 0.0$

CPM values with different superscripts indicate a significant difference at $P < 0.05$.

[*] One mortality occurred at 15 d post-challenge. Clinical signs of CWD were not present; however, a few yellow-pigmented bacterial colonies were re-isolated from spleen tissue.

Table 2
Cumulative percent mortality (CPM) ± standard error of the mean (SEM) and relative percent survival (RPS) among rainbow trout following *F. psychrophilum* (CSF-259-93) challenge at 8 and 15 weeks post-immunization

Treatment	Challenge time*	Challenge dose (cfu fish^{-1})	Mean CPM ± SEM and (RPS)	Mean titer ± SEM
PBS	8	2.1×10^6 4.5×10^6	$98.7^a \pm 1.3$ $97.3^a \pm 2.7$	$<100^a$
259-93B.17	8	2.1×10^6 4.5×10^6	$54.1^b \pm 2.1$ (45.2) $67.1^b \pm 2.5$ (31.1)	$1400^b \pm 570$
PBS	15	1.8×10^6	$96.0^a \pm 2.3$	$<100^a$
259-93B.17	15	1.8×10^6	$68.0^b \pm 2.3$ (29.2)	$285^b \pm 94$

Fish were immunized by intraperitoneal injection with the 259-93B.17 strain or mock immunized with phosphate buffered saline (PBS) and mean antibody titers ± SEM were determined prior to each challenge time. Within each challenge dose or time, mean CPM and titer values were compared, respectively; values with different superscripts indicate a significant difference at $P < 0.05$.

* Weeks post-immunization.

15 weeks post-immunization ($P < 0.05$). Relative percent survival values of up to 45% were observed (Table 2).

Challenge mortalities exhibited typical signs of *F. psychrophilum* infection. Yellow-pigmented bacteria phenotypically characteristic of *F. psychrophilum* were re-isolated from 98% (96/98) and 95% (52/55) of the mortalities examined at the 8 and 15 weeks post-immunization challenges, respectively. There were no mortalities in the mock infected control groups.

Immunization of rainbow trout with the 259-93B.17 strain elicited specific antibody responses against *F. psychrophilum* as determined by ELISA. There were no significant differences in the serum antibody titers between treatment and control fish ($P > 0.05$) at the time of booster immunization. At 8 and 15 weeks post-immunization, there were significant differences in mean serum antibody titers between fish immunized with the 259-93B.17 strain and those injected with PBS ($P < 0.05$). Fish immunized with the 259-93B.17 strain exhibited mean antibody titers (± standard error of the mean) of 1400 ± 570 and 285 ± 94 at 8 and 15 weeks post-immunization, respectively. Specific antibodies were not detected in any of the pooled serum samples from fish injected with PBS (titer < 100).

3.5. Immersion immunization trial

Immunization of rainbow trout by immersion with the 259-93B.17 strain also conferred protective immunity following bacterial challenge with *F. psychrophilum* at 10 weeks post-immunization (Table 3). At the low challenge dose (3.3×10^5 cfu fish^{-1}), the CPM of immunized fish (without adipose fin removal) was lower than the CPM of the mock immunized control group, but this difference was not statistically significant ($P = 0.054$). Removal of the adipose fin prior to primary-immunization enhanced protective immunity, and the CPM of fish in this group was significantly different than that of the mock immunized control group ($P < 0.05$) but not significantly different than the CPM of immunized fish that did not have adipose fins removed ($P > 0.05$). Relative percent survival values of 45% and 28% were observed for fish treated with or without adipose fin removal prior to immunization, respectively. At the high challenge dose (2.0×10^6 cfu fish^{-1}) there were no significant differences among treatment and control groups (Table 3).

Table 3
Cumulative percent mortality (CPM) ± standard error of the mean (SEM) and relative percent survival (RPS) among rainbow trout following *F. psychrophilum* (CSF-259-93) challenge at 10 weeks post-immunization

Treatment	Adipose fin removal	Mean CPM ± SEM and (RPS)	
		3.3×10^5 cfu fish^{-1}	2.0×10^6 cfu fish^{-1}
TYES	No	$72.5^a \pm 2.5$	$80.0^a \pm 0.0$
259-93B.17	No	$52.5^{a,b} \pm 2.5$ (28)	$77.5^a \pm 7.5$ (3)
259-93B.17	Yes	$40.0^b \pm 5.0$ (45)	$85.0^a \pm 10.0$ (0)

Fish (with or without adipose fin removal) were immunized by immersion with the 259-93B.17 strain or mock immunized by immersion into water containing TYES. CPM values with different superscripts indicate a significant difference at $P < 0.05$.

Challenge mortalities exhibited typical signs of *F. psychrophilum* infection and yellow-pigmented bacteria phenotypically characteristic of *F. psychrophilum* were re-isolated from 95% (76/80) of the mortalities examined. There were no mortalities in the mock infected control groups.

4. Discussion

This study describes the development and characterization of two rifampicin resistant strains of *F. psychrophilum* that exhibit reduced virulence in rainbow trout when compared to the parent strain from which they were generated. The 259-93B.17 strain exhibited a high level of attenuation, whereas the 259-93A.16 strain was modestly attenuated and retained the ability to cause disease. This is in accordance with previous research that has demonstrated rifampicin resistant strains of pathogenic bacteria may exhibit a loss or reduction of virulence [18,28,34–37].

The mechanism by which rifampicin resistance leads to the attenuation of bacteria is not known and may be variable between strains. Rifampicin is a broad-spectrum antibiotic that inhibits bacterial DNA-dependent RNA polymerase by binding to the β subunit, thereby blocking transcriptional initiation [33]. Resistance to rifampicin arises from mutations in the *rpoB* gene that encodes the β subunit of the RNA polymerase [46–48]. Interestingly, mutations in the *rpoB* gene of different bacterial species can cause global effects on growth and developmental events [49], reduced fitness [50–52], and other altered phenotypes [53–55]. The pleiotropic nature of phenotypes exhibited by rifampicin resistant strains of bacteria suggests that the expression of numerous genes can be affected. Thus, one hypothesis for attenuation is that the RNA polymerase mutations induced by rifampicin affect its activity and global gene expression.

Another hypothesis for the attenuation of rifampicin resistant bacterial strains is that rifampicin induces mutations into genes involved in the biosynthesis of the *O*-polysaccharide (*O*-PS) component of LPS. An attenuated rifampicin resistant strain of *F. columnare* was produced [28] and analysis of this mutant showed that it lacked high molecular mass LPS, indicating a probable mutation in genes involved in the biosynthesis of the *O*-PS [56]. Schurig et al. [37] developed a rifampicin resistant strain of *B. abortus* (RB51) that lacked expression of the O-PS or produces low levels of *O*-PS [57]. Vemulapalli et al. [58] demonstrated that the RB51 strain contains a disruption in the *wboA* gene responsible for the synthesis of the *O*-PS that was not present in the parent strain and the RB51 strain may possess other mutations in genes involved in the synthesis

of *O*-PS [59]. Klesius and Shoemaker [18] developed an attenuated rifampicin resistant strain of *E. ictaluri* (RE-33) and found that it expresses short chains of *O*-PS, but does not express LPS molecules of high molecular mass consisting of numerous *O*-PS repeat units [60]. Interestingly, no mutations were detected in LPS biosynthesis genes of the resistant mutant [60]. The *O*-PS of *B. abortus* and *E. ictaluri* are generally accepted as important virulence factors of these pathogens [31,61], and the lack of normal expression of the *O*-PS likely accounts for the attenuated phenotypes of the rifampicin resistant strains. It remains to be determined whether rifampicin directly induces mutations in genes involved with LPS biosynthesis or if the expression of these genes is affected by altered RNA polymerase activity induced by rifampicin. A third hypothesis is that the attenuated phenotype is unrelated to rifampicin resistance and may represent spontaneous mutations occurring from serial passage of the bacterium *in vitro*.

In the present study, the parent and rifampicin resistant *F. psychrophilum* strains were characterized to attempt to determine the basis for the attenuation observed. The LPS banding patterns of the rifampicin resistant strains were compared to the parent strain but there were no apparent differences. Similarly, Bhatnagar et al. [34] showed that rifampicin resistant strains of *Francisella tularensis* had LPS banding patterns identical to parent strains. These observations demonstrate that the loss of *O*-PS expression is not always associated with rifampicin resistance.

Growth of the rifampicin resistant strains was slower than the parent strain, which is consistent with other studies [36,51,53]. Because both strains had reduced growth rates, but only one (259-93B.17) was highly attenuated, it appears that growth was not necessarily associated with the differences in virulence. However, the 259-93B.17 strain exhibited five protein differences in the whole-cell lysate profiles when compared to the parent and 259-93A.16 strains, which may suggest that gene expression was affected by the induction of rifampicin resistance in this strain. Interestingly, three of these differences involved increased expression while two cases involved reduced expression. The identity and function of these proteins has not been determined. It is possible that these specific proteins are involved in attenuation or they signal alterations in gene regulatory networks that produce an inappropriate response (attenuation) *in vivo*. Further research is needed to determine the basis of attenuation observed in the 259-93B.17 strain.

Attenuated strains produced by selecting for resistance to rifampicin have been used successfully as live attenuated vaccines [18,28,37,62,63]. Similarly, immunization of rainbow trout by ip injection with the rifampicin resistant 259-93B.17 strain conferred a protective immune response against *F. psychrophilum* challenges. Although challenge of control fish at two different doses resulted in similar and high CPM (nearly 100%), there was a dose response in the protection of immunized fish following challenge at these doses. The RPS value of immunized fish at the high challenge dose was 31%, while the RPS value at the low challenge dose was 45%. Thus, it is surmised that higher levels of protection would be evident if the challenges resulted in a more typical mortality rate (~60%) in the control groups [44]. Another consideration is the unnatural route of infection induced by injection challenge, and it is possible that protection would be greater following a natural challenge. Importantly, at 15 weeks post-immunization, fish immunized with 259-93B.17 exhibited an RPS of 29%, indicating that the results were reproducible at later time points.

A practical delivery method, such as immersion, is one important consideration for the feasibility of a vaccine for many aquaculture species susceptible to *F. psychrophilum*. To address this, a pilot study was performed and the results demonstrated that protective immunity was conferred to rainbow trout immunized by immersion with the live attenuated 259-93B.17 strain following challenge with the low dose of *F. psychrophilum* at 10 weeks post-immunization. Removal of the adipose fin prior to immunization enhanced this protective immune response and an RPS of 45% was obtained. This treatment was included because past research demonstrated that immersion challenge models with *F. psychrophilum* are inconsistent at inducing infection unless a portal of entry is provided [3,64–68]. The results of the present study suggest that the 259-93B.17 strain behaves similarly; thus, providing the bacterium with a portal of entry enhanced colonization and subsequent immune responses. However, protection (29% RPS) was also conferred to fish immunized with the 259-93B.17 strain (without adipose fin removal), which clearly demonstrates the potential for the use of this *F. psychrophilum* strain as a live attenuated vaccine. Protection was not demonstrated in immunized fish following challenge at the high dose of *F. psychrophilum*, which suggests this dose overwhelmed the protective immune responses that were elicited. Research efforts should focus on optimizing immersion immunization with the 259-93B.17 strain and evaluating other delivery methods that promote the uptake of bacterial cells for the enhancement of protective immune responses.

The protection obtained in this study is also of considerable interest because previous research has demonstrated that immersion and ip injection immunization of rainbow trout with formalin-killed *F. psychrophilum* cells does not provide protection following challenge unless an adjuvant is used and fish produce elevated antibody titers [5]. Although specific antibody against *F. psychrophilum* is correlated with protection [8,10, 12,43], research has also suggested that other immune components may be necessary for complete protection [5,43,69]. In the present study, fish immunized with the 259-93B.17 strain by injection exhibited significantly elevated antibody titers at 8 and 15 weeks post immunization. Specific antibody present in immunized fish may have contributed to the protection observed, but it should be noted that levels of circulating antibody decreased between 8 and 15 weeks post-immunization. Further, the antibody titers detected in this study were relatively low compared to past studies [5,11]. Since the ELISA assay used antigens from the parent CSF-259-93 strain, it is possible that important epitopes were not available and this resulted in an artificially lower titer. This is further supported by the observation that the 259-93B.17 strain exhibits differential protein expression compared to the parent strain. In addition to the induction of specific antibody responses, it is also possible that immunization with the 259-93B.17 strain stimulated other components of the immune system and these live cells may have adjuvant-like properties that enhanced protection. Previous research has demonstrated that immunization of fish with live attenuated bacterial vaccines induce specific cellular and humoral immune responses [17,70–72] as well as non-specific humoral immune components such as lysozyme and antiprotease activity [72]. Future research should further define the specific and non-specific immune responses induced by the 259-93B.17 strain to understand the mechanisms by which this live attenuated strain induces protection from *F. psychrophilum* infections.

Recently, Alvarez et al. [30] reported on a mutant strain of *F. psychrophilum* that was isolated by transposon mutagenesis. The mutant contained a disruption in the *exbD2* gene of the TonB system, which is involved in the acquisition of iron from the environment, and this mutant was shown to be attenuated in rainbow trout. Immunization of rainbow trout by ip injection with the mutant strain conferred protection against a low challenge dose of the virulent parent strain [30]. Following challenge, the authors observed that lesions developed on both the immunized and control fish; however, the lesions on the immunized fish tended to heal over time. In the present study, injection and immersion immu-

nization of rainbow trout with the rifampicin resistant 259-93B.17 strain resulted in protective immunity against *F. psychrophilum* challenge, and similar observations of lesion healing were noted in the immunized fish. Taken together, the results of these studies demonstrate that the use of attenuated strains of *F. psychrophilum* is a rational approach for vaccine development.

In summary, an attenuated strain of *F. psychrophilum* (259-93B.17) was developed by selecting for resistance to the antibiotic rifampicin and immunization of rainbow trout with this strain by injection and immersion delivery stimulated long lasting protective immune responses. The results demonstrate that the rifampicin resistant 259-93B.17 strain shows promise as an effective live attenuated vaccine for the prevention of CWD.

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